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STUDIES OF FERTILIZATION.

IX. On the Question of Superposition of Fertilization on Parthenogenesis in Strongylocentrotus purpuratus.

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The statement of Loeb (1913, p. 234; 1915, p. 260–261) that eggs of *Strongylocentrotus purpuratus* which have formed membranes as a result of butyric acid treatment can be fertilized with sperm if the membranes are destroyed by shaking raises a difficult question in the problem of fertilization. It is known that eggs in which membranes have been formed by spermatozoa are incapable of refertilization, even if the membranes are destroyed by shaking immediately after their formation. We have every reason to believe that the membrane-forming reaction by butyric acid is the same as by fertilization. The after effects should therefore be the same.

In an examination of this problem in the case of *Arbacia,* C. R. Moore (1916) showed that this is the case, viz: that, given a full membrane reaction by butyric acid, the eggs became incapable of fertilization, even if the membranes are removed. It is, however, possible to superimpose fertilization on an *incomplete* reaction caused by butyric acid to a variable extent which is roughly proportional to the degree of the original reaction. Just (1919) investigated the same problem in the case of *Echinarachnius parva,* and determined that eggs which have formed full membranes after butyric acid treatment do not respond to subsequent insemination whether the membranes are removed or not.

The writer took advantage of the opportunity afforded by a stay at the Hopkins Marine Station in Pacific Grove, California, in January and February, 1920, to repeat Loeb's experiments on the same species that he used. The results obtained diverged

considerably from Loeb's account and they are therefore recorded for the purpose of clearing up the apparent flat contradiction between the results of Loeb on *Strongylocentrotus purpuratus* and of Moore and Just on other forms.

The conditions of activation by butyric acid should first be recalled. It is convenient to proceed from a standard concentration of butyric acid, and accordingly the strength used by Loeb was adopted for all the experiments, viz: 2.8 c.c. N/10 butyric acid + 50 c.c. sea-water. Membranes are not formed in butyric acid, but only after transfer of the eggs to sea-water. The optimum time of exposure to butyric acid for membrane formation in sea-water is a function of temperature; a series of determinations showed the following approximate optimum exposures: 15° C., 110–120 seconds; 17°, 90 seconds; 20°, 80 seconds; 24.5°, 40 seconds; 30°, 18 seconds. It is therefore very important that the temperature of any given experiment should be known; the temperature of the experiments recorded here was always close to 16° C. The optimum time of exposure is also rather narrowly limited at any temperature. Thus in one experiment where 105 seconds was optimum, 90 seconds was too short and 120 seconds too long for optimum results.

The concentration of the butyric acid to which the eggs are exposed is another vital factor; therefore in adding the eggs to the butyric acid the same amount of sea-water should be carried over with them each time; in my experiments this was 2.5 c.c. The membrane reaction after transfer to sea-water depends on removal of the action of the butyric acid; it is therefore important not to carry over too much butyric acid in making this transfer; the transfers were therefore made uniformly to 100 c.c. sea-water and not more than 2.5 c.c. of the butyric acid solution was carried over. The eggs were not fertilized in this sea-water, but from 2 to 5 drops were transferred to 10 c.c. of fresh sea-water in a Syracuse watch crystal in which the inseminations were made; thus the butyric acid was reduced to a negligible quantity in the insemination.

Considerable variations in temperature or of conditions of transfer to or from the butyric acid will produce considerable

variations in results. But if an adequate series of controls is kept it is possible to check these up.

The following controls were kept: (1) Some untreated eggs. (2) Eggs left in the butyric acid. (3) Eggs left in the sea-water to which transfer was made from butyric acid (unfertilized). (4) When eggs were shaken for superimposed insemination, some were left unfertilized. (5) In some cases samples from control 3 were fertilized without shaking. With these controls one can follow back the history of any given result and determine the percentage and kind of membranes originally formed, the extent to which they were destroyed by shaking, and the capacity for fertilization without shaking.

The eggs used for any given experiment were selected usually from several lots representing different females; those eggs that gave the best reaction to insemination being selected.

As Loeb and others have noted, the eggs that have received the butyric acid treatment do not segment without subsequent treatment, but invariably cytolyze, or die from other causes, sooner or later. This happened invariably in my controls (3) and not a single segmenting egg was recorded from such controls.

The membranes when first formed are soft, and are easily destroyed by shaking within two minutes (approximately) after transfer to sea-water from the butyric acid; but they soon harden and thereafter it is difficult to destroy them by shaking. Moreover, the membranes do not form instantaneously after transfer to sea-water, but require about the same time (approximately 30 seconds at 15° C.) as for their formation by insemination. The time for effective results from shaking is therefore cut down accordingly.

The exposure to butyric acid thus acts like insemination only in the sense that it prepares the egg for membrane formation, but the reaction does not begin until the butyric acid is removed; and, after that, the time required for membrane formation is the same as for membrane formation of control eggs by insemination. So long as the eggs are in butyric acid, therefore, they presumably maintain a fertilizable condition, and it requires about 30 seconds after transfer for this fertilizable condition to become lost by occurrence of the full membrane reaction.

If the exposure to butyric acid is not sufficient to produce the membrane reaction after transfer to sea water the eggs are mostly fertilizable as though they had not been exposed to the acid. If the reaction after transfer to sea-water is incomplete, so that membranes are not fully formed, some eggs retain a certain amount of capacity for fertilization after the membranes are removed so that they may segment; they do not, however, proceed far with the developmental process under such circumstances, rarely to the gastrula stage. Moreover, the eggs of any lot are variable, within rather narrow limits, with respect to the degree of reaction after any given exposure. It would therefore hardly be expected that all eggs of any given lot would give the optimum membrane reaction after any given exposure, and one would expect a certain minimal capacity for segmentation after superimposed insemination with the best possible lot of eggs.

Even in Loeb's experiments the outstanding fact is not the one that he emphasizes, viz: that a certain percentage of eggs may segment after superimposed fertilization when the membranes are removed, but that most of the eggs do not do so. Loeb reports as high as 20 per cent. segmentation after such a superimposed insemination; 80 per cent. of the eggs even in this case had become unfertilizable, and this is the significant result. I have never obtained such a high percentage of segmenting eggs in any of my experiments after the optimum exposure, and my conclusion is that in this particular experiment of Loeb's the eggs were on the whole under-exposed to butyric acid. I am strengthened in this opinion by the fact that he reports that the developing eggs formed *normal larvæ* (1915, p. 261), a result that is never obtained, in my experience, after an optimum exposure. The experiment in question is reported as though only one exposure time were used, in which case it would be a matter of good fortune to hit the exact exposure time. In my experiments at least three exposure times were always used by transferring lots of eggs to sea water at 15 second intervals before, at, and after the estimated time of optimum exposure; then treating the entire series.

Reference should be made to the tabulated results of the principal experiments (pp. 29–31) for the following discussion:

It will be seen from experiments 2 and 7 that at 15–16° C. the unshaken eggs have practically full fertilization capacity up to a 30-second exposure to butyric acid and that the transition to the unfertilizable condition is very sudden—thus between 30 and 45 seconds (Exp. 7) and between 30 and 60 seconds (Exp. 2). This transition coincides with the onset of membrane formation and all eggs that have formed membranes fail to fertilize. This is well known from previous experiments, but it is not generally recognized that many eggs that fail to form membranes also become unfertilizable. Thus in experiment 2*c* after 60 seconds exposure to butyric acid 15 per cent. of the eggs failed to form membranes, but only 4.9 per cent. of all eggs segmented after insemination. In experiment 7 this comes out even more clearly: 30 per cent of the eggs failed to form membranes in 7*b* (after 45 seconds exposure) and only 0.2 per cent. of all eggs segmented after insemination.

If the eggs be shaken within about 2½ minutes after transfer to sea-water the soft membranes are destroyed; there is however a rapid transition from this soft condition of the membrane to a hard condition in which it is difficult to destroy the membranes by shaking. Rather complete destruction of membranes in the soft condition is accompanied by extensive agglutination of the eggs, some, however, remaining free. Agglutination of the eggs is indeed a fair index of the degree to which the membranes have been destroyed.

When the membranes are thus destroyed there is a slight increase in the percentage of eggs that segment after insemination as compared with unshaken eggs (cf. Exp. 2, *d, e;* Exp. 3; Exp. 8). The percentage is in any case exceedingly small, and the impressive fact is that in all cases over 95 per cent. of the eggs, indeed generally over 99 per cent. of the eggs, are incapable of segmenting after insemination. Although the percentage is so small it must, I think, be admitted as possible that there is a small percentage of eggs that have formed membranes that still possess the capacity of segmenting after insemination. In other words, I think that this small percentage of segmenting eggs is not necessarily to be referred to eggs that failed in the membrane reaction,

for such eggs should fertilize without shaking, and this they fail to do as the unshaken inseminated control shows. The eggs that segment do not form a new membrane and they fail to develop far; in the loose type of cleavage and irregularity of development they exhibit a weak condition.

The rather significant fact will be noted in the tables that the small percentages of segmenting eggs involved diminish in each experiment with increased time of exposure: thus in Exp. 2: 0.6 per cent. after 1½ minutes exposure, < 0.1 per cent. after 2 minutes exposure; Exp. 3: 1.3 per cent. after 1½ minutes, 0.15 per cent. after 1¾ minutes, and 0.25 per cent. after 2 minutes (the slight increase here is not significant, and is probably due to sampling); Exp. 8: 0.3 per cent., 0.15 per cent. and 0 after 1¼, 1½ and 1¾ minutes respectively; Exp. 9: 4 per cent., 1.5 per cent., 0.3 per cent. after 2, 2¼ and 2½ minutes respectively. (In Exp. 9 it will be noted in the protocol that membrane formation was not very complete.) There is a tendency in each experiment towards a vanishing point. This can mean only that each egg tends towards an unfertilizable state under the conditions of the experiment and that the small residuum shown in the experiments is due to the statistical conditions of individual variation.

In each experiment the eggs were very heavily inseminated, 10 to 20 times as much sperm being used as required for normal fertilization. The spermatozoa did not, however, penetrate into the experimental eggs except in rare cases, as shown by sections. In those cases in which they were observed in sections to have entered, apparently usually at points of injury to the egg-surface, the spermatozoa remained unaltered in the cytoplasm. The yet rarer cases in which a reaction resulting in cleavage was set up in the egg were not observed in the sections, for the painstaking search necessary to find them in such a mass of material did not seem worth while.

My results agree in principle with those of Moore and Just and are in disaccord with those of Loeb. It will be noted from the tables that the optimum time of exposure to butyric acid varies considerably even at the same temperature with different lots of

eggs. Failure to take this into account may make considerable difference in the results of superimposed insemination. The fact that Loeb secured 20 per cent. of *vigorous* larvæ in the principal experiment that he cites suggests an under exposure, for the eggs inseminated after the optimum exposure to butyric acid, that segment, never develop normally.

Conclusion.—The membrane reaction after butyric acid treatment is the same as after insemination; this is shown by similarity of the membranes formed in the two cases, and by the fact that the rate of formation is the same. The result of rendering the egg insusceptible to spermatozoa shows a possible slight difference only, and this receives an obvious explanation on the assumption that there is a variable tendency towards incompleteness of reaction after butyric acid which in rare cases leaves some of the reacting substances of the egg (fertilizin) free for sperm action.

EXP. 2. TEMPERATURE ESTIMATED 15–16° C.

Exposure to Butyric.	Fertilized Without Shaking.	Shaken After Membrane Formation.	Fertilized after Shaking.
a. 15 secs.....	99+%	15 min.	78% segmented
b. 30 "	98 %	15 "	80% segmented
c. 60 "	4.9%	15 "	0.7% segmented
d. 90 "	0	1½[1] min.	0.6% segmented
e. 120 "	0	2[1] "	<0.1% segmented
f. 150 "	0		
g. 240 "	0		
h. 360 "	0		

Membranes formed as follows after butyric: *a*, 0; *b*, 0; *c*. 85 per cent.; *d*, 99 per cent. (1 per cent. without); *e*, 99 per cent. (optimum); *f*, 99 per cent. (some imperfect); *g*, 99 per cent. (imperfect); *h*, 99 per cent. (imperfect).

It is doubtful if any membranes were destroyed by shaking 15 minutes after their formation (*a*, *b*, *c*). In *d* 75 per cent. of the membranes were destroyed.

[1] These represent a sample of the same lot of eggs tested two hours later than all other items of this experiment.

EXP. 3. TEMPERATURE ESTIMATED 15–16° C.

Exposure to Butyric.	Fertilized Unshaken.	Shaken After Membrane Formation.	Fertilized after Shaking.
a. 90 secs.....	0 segmented	$1\frac{1}{2}$ min.	1.3% segmented
b. 105 "	0 "	$1\frac{3}{4}$ "	0.15% "
c. 120 "	0 "	$2\frac{1}{4}$ "	0.25% "

a. Membranes generally excentric, many partial.

b. All have fine membranes (optimum).

c. All have membranes, generally good, some excentric.

In *a* all membranes destroyed; eggs agglutinated.

In *b* 70 per cent. membranes destroyed; much agglutination.

In *c* 50 per cent. membranes destroyed; much agglutination.

EXP. 7.[1] TEMPERATURE 15° C.

	Exposure to Butyric.	Membranes.	Fertilized Unshaken.
a.....	30 secs.	0	95% segmented.
b.....	45 "	70%	0.2% "
c.....	60 "	99%	0 "
d.....	75 "	99%	1 egg "
e.....	90 "	99%	
f.....	90 "	100%	0
g.....	105 "	100%	0
h.....	120 "	99% (excentric)	0
i.....	135 "	98% "	0
j.....	150 "	100% ? (excentric)	0
k.....	180 "	100% ? "	0
l.....	240 "	100% ? "	0
m.....	360 "	99% "	0
n.	600 "	" "	0
o.....	1200 "	10%	0

[1] This experiment was run in two sections, *a–e* and *f–o*. The exposure times *y*, *e* and *f* were thus repeated.

EXP. 8. TEMPERATURE 17° C.

	Exposure to Butyric.	Fertilized Unshaken.	Shaken After Membrane Formation.	Fertilized after Shaking.
a...	75 secs.	No cleavage	2.5 min.	0.3% segmented
b...	90 "	" "	3.5 "	0.15% "
c....	105 secs.	" "	4 "	0 "

All of *a*, *b* and *c* formed membranes.

Shaking destroyed 50 per cent. membranes in each case.

EXP. 9. TEMPERATURE 16.5° C.

	Exposure to Butyric.	Shaken After Membrane Formation.	Fertilized after Shaking.
a.....	120 secs.	2 min.	4% segmented
b.....	135 "	2 min. 35 secs.	1.5% "
c.....	150 "	3 " 20 "	0.3% "

In *a* only 50 per cent. formed full membranes (under-exposure).
In *b* 95 per cent. formed full membranes.
In *c* 50 per cent. formed full membranes (over-exposure).
In *a* shaking destroyed all memberane.
In *b* shaking destroyed 50 per cent. of the membranes.
In *c* shaking destroyed 20 per cent. of the membranes.

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